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Mechanistic and Metabolic Studies of Sterol 24,25-Double Bond Reduction in *Manduca sexta*

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Larvae of *Manduca sexta* were used to obtain a cell-free sterol 24,25-reductase. From the midgut of fifth instar larvae fed a mixture of sitosterol and campesterol a microsome-bound 24,25-sterol reductase was prepared that transformed desmosterol (K_m, 3 μM), lanosterol (K_m, 18 μM), and cycloartenol (K_m, 33 μM) to cholesterol, 24,25-dihydrolanosterol, and cycloartanol, respectively. With desmosterol as substrate, the microsome-bound enzyme was found to incorporate tritium into cholesterol from 4S-tritium labelled NADPH. [24-^{2}H]lanosterol was transformed by larvae to [24-^{2}H]24,25-dihydrolanosterol (structure confirmed by mass spectroscopy (MS) and ^{1}H-nuclear magnetic resonance spectroscopy. A rationally designed inhibitor of 24,25-reductase activity, 24(*R,S*),25-epiminolanosterol (IL), was assayed and found to be inhibitory with an I_{50} of 2 μM. IL was supplemented in the diet of *M. sexta* with either sitosterol or stigmasterol and found to inhibit development (I_{50}, 60 ppm). The major sterol which accumulated in the IL-treated larvae was desmosterol, confirming the site of inhibition was reduction of the 24,25-bond. IL was converted to [2-^{3}H]IL when fed to the larvae. [2-^{3}H]lanosterol was recovered from fifth instar larvae and its structure confirmed by MS and radiochemical techniques.

Key words: ***Manduca sexta*, tobacco hornworm, sitosterol, desmosterol, cholesterol**

INTRODUCTION

Insects, unlike mammals, are unable to synthesize sterols de novo and therefore acquire sterols from their diet or from symbionts (Kircher, 1982; Maurer et al., 1992). It is of interest to note this dietary requirement for sterol represents the only proven difference in nutritional requirements between insects

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and mammals (Svoboda and Chitwood, 1992; Grieneisen, 1994). Species such as the tobacco hornworm, *Manduca sexta,* satisfy their sterol requirement by feeding on tobacco plants (and related plants). In the field, they utilize a variety of Δ^5-phytosterols after C-24 dealkylation of the sterol side chain to produce cholesterol. The pathway for the transformation of sitosterol (24α-ethylcholesterol)—together with stigmasterol (24α-ethylcholesta-5,22-dien-3β-ol), it is the predominant phytosterol in tobacco (Nes et al., 1982) and related vascular plants (Patterson, 1994)—to cholesterol by the tobacco hornworm has been established (Svoboda, 1994). Figure 1 shows the stereochemistry at C-24 and C-25 of sitosterol (Nes et al., 1992) and its relationship mechanistically to C-24 dealkylation of phytosterols (Ikekawa, 1993). Cholesterol functions in two roles in *M. sexta:* as a membrane insert and as a precursor to the C_{27} ecdysteroids (moulting hormones) (Kircher, 1982). The physiological necessity for cholesterol to be turned over to ecdysteroids was shown by incubation with azasteroids, which induced precocious fourth instar prepupa and abnormal prepupal-pupal forms (Svoboda et al., 1972). The primary mechanism of action of the azasteroids was found to result from inhibition of desmosterol (cholesta-5,24-dien-3β-ol) conversion to cholesterol (cholest-5-en-3β-ol). Thus inhibition of phytosterol metabolism to cholesterol by interfering with Δ^{24}-sterol reductase activity presents a unique target site that might be exploited further as a selective, biorational insect-control technology.

The terminal enzyme step of phytosterol transformation to cholesterol presumably involves saturation of the 24,25-bond by a NADPH-linked sterol reductase (Fig. 1). It is assumed that the mechanism of reduction of the 24,25-bond by insects is similar to the transformation performed by mammals (Fujimoto et al., 1974, 1991). Chemically, the carbon-carbon double bond reduction should involve a *trans*-directed Markovnikov addition that proceeds through a transition site intermediate that produces a C-25 carbenium ion (Wilton et al., 1970). Because the azasteroids (a related side chain containing N-sterols [Janssen and Nes, 1992]) are thought to be protonated at physiological pH, they should be in a chemical form to mimic the transition state of the reaction progress involving reduction of the 24,25-bond. The initial addition of a proton to C-24 is thought to come from water, whereas the C-25 carbenium ion may be neutralized by delivery of a hydride ion from the NADPH. The stereochemistry of attack of the incoming H^+/H^- is now assumed to be by the *cis* addition of the two hydrogen atoms to C-24 and C-25, respectively (Kienle et al., 1973). Fujimoto et al. (1991) have demonstrated the stereochemistry of the hydrogen addition to C-25 of desmosterol by the sterol-Δ^{24}-reductase of the silkworm, *Bombyx mori,* using ^{13}C-labeled sterol substrate and ^{13}C-NMR* spectroscopy. Whereas a cell-free preparation of the Δ^{24}-sterol reductase has been reported for *M. sexta* (Svoboda et al., 1969) and *B. mori* (Fujimoto et al., 1991), there have been no efforts to determine the sterol

*Abbreviations used: GLC = gas liquid chromatography; HPLC = high-pressure liquid chromatography; IL = iminiolanosterol; MS = mass spectroscopy; NLF = nonsaponifiable lipid fraction; NMR = nuclear magnetic resonance spectroscopy; RRT_c = relative retention time, relative to cholesterol; TLC = thin layer chromatography.

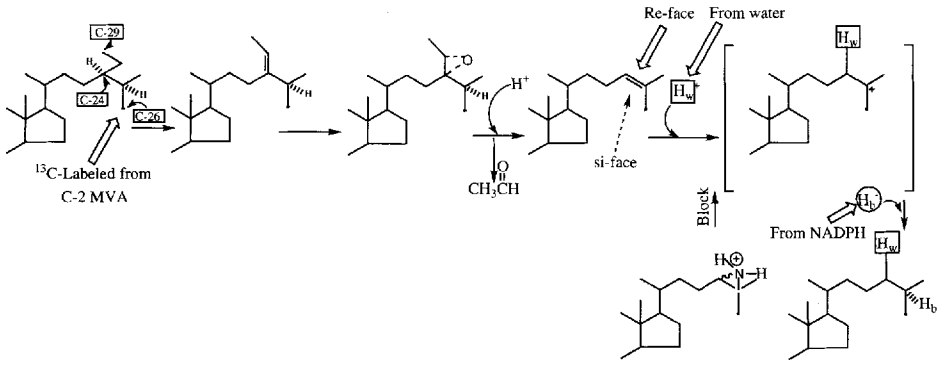

Fig. 1. Hypothetical pathway of sitosterol C-24 dealkylation to cholesterol and interference by 24(*R,S*),25-epiminolanosterol. Note the inhibitor is in the salt form which mimics the transition state intermediate formed by the native sterol substrate during catalysis.

specificity for binding/catalysis to the enzyme. Most of the earlier studies involved rearing insects on different sterols and with inhibitors to study sterol utilization and metabolism (Ikekawa et al., 1993; Fujimoto et al., 1985; Svoboda, 1994).

Therefore, the purpose of this study was to provide information on the sterol substrate specificity of the Δ^{24}-sterol reductase from *M. sexta*, examine the mechanism of sterol 24,25-reduction operating in *M. sexta*, and test in vivo and in vitro a novel ecdysteroid biosynthesis inhibitor designed to interfere with sterol 24,25-reduction, 24(*R,S*),25-epiminolanosterol (IL).

MATERIALS AND METHODS

Manduca sexta (L)

Larvae of *M. sexta* were reared at the USDA laboratory at Beltsville, MD, on artificial diets which contained 340 ppm sterol; 100 ppm phytosterol (mostly 24-alkyl sterol; see Results) was from wheat germ (a necessary component of the diet), and 240 ppm test sterol was from coating the dry diet ingredients with sitosterol or stigmasterol dissolved in dichloromethane. As shown earlier, the phytosterol in the wheat germ was below the minimum concentration required for sterol-promoted maturation of the insect (Svoboda and Robbins, 1968; Svoboda, 1994). Sitosterol and stigmasterol were isolated from natural sources and their structures confirmed by ^{1}H-NMR (Guo et al., 1995). IL and [2-^{3}H]IL were synthesized from lanosterol as described (Le and Nes, 1986; Nes et al., 1989). Test sterols and IL were supplied to the USDA laboratory for incubation with *M. sexta*. IL was included in the diet at increasing concentrations from 1 to 520 ppm in order to observe possible effects on sterol metabolism, larval growth, and development. Three- and five-day-old fifth instar larvae were examined for sterols, and three-day-old fifths were used in the enzyme studies. Insects reared at Beltsville were harvested in batches of five or six, weighed, and either stored and shipped in MeOH for sterol analysis or sent live to W.D.N. by overnight mail express (usually two batches per week). Upon receipt, live insects (the source for en-

zyme study) were cooled on ice and then dissected in a small amount of saline solution to remove midguts as reported (Svoboda et al., 1969). The midguts were the source of the microsome-bound Δ^{24}-sterol reductase. The insects were stored at –20°C prior to sterol analysis.

Assay of Effects of the Test Compounds Upon Sterol Metabolism in Tobacco Hornworm

The sterols were isolated from tobacco hornworm prepupae and analyzed qualitatively and quantitatively by TLC, GLC, and HPLC according to established methods (Guo et al., 1995; Nes et al., 1991; Xu et al., 1988). By comparison of the relative percentages of cholesterol and desmosterol in the sterol mixture from control and IL-treated insects, the degree of dealkylation of sitosterol by the insect and inhibition of the Δ^{24}-sterol reductase enzyme was established. Confirmation that IL attacked the Δ^{24}-sterol reductase specifically was by an in vitro study using a preparation of a microsome-bound enzyme that was capable of transforming desmosterol to cholesterol.

Sterol Analysis

Each group of insects was saponified with 10% KOH in 90% aq. MeOH at reflux temperature for 30 min. The neutral lipids obtained by dilution with water and extraction with diethyl ether were chromatographed on TLC in benzene-ether (85/15). The sterols were separated by the degree of substitution at C-3 into 4-desmethyl, 4-monomethyl, and 4,4-dimethyl sterols; sterols were eluted from the plate corresponding to the three sterol classes (Xu et al., 1988). They were quantified and identified by GLC using a 3% SE-30 packed column operated isothermally at 245°C. GC-MS was performed on a tabletop GC-MS (Hewlet Packard, San Fernando, CA) 5890 series II gas chromatograph coupled to a 5970 mass detector operating at 70 ev) as described (Guo et al., 1995). HPLC was performed using several reversed-phase columns which we recently demonstrated have different sterol resolution properties (Guo et al., 1995; Xu et al., 1988). Retention times in GLC (RRT_c) and HPLC (α_c) are relative to the retention times of cholesterol. The ^{1}H-NMR was performed on pure samples resolved by HPLC. Samples were dissolved in $CDCl_3$. The spectra were obtained at Texas Tech University on either a Bruker (Billerica, MA) AF-300 or AF-200 NMR spectrometer with trimethylsilane (TMS) as the reference standard.

Synthesis of ^{2}H and ^{3}H Substrates

Nonradioactive sterols used as reactants for the preparation of [3-^{3}H]-sterols have been isolated from natural sources or were commercially obtained (then purified by HPLC), as described in our earlier publications (Guo et al., 1995; Nes et al., 1991). The 3-keto sterol was converted to the corresponding 3β-OH sterol by reduction with sodium borotrituride (1,000 mCi/mmol; NEN, Boston, MA) and purified by HPLC to remove the 3α-epimer and side products, as described (Le and Nes, 1986). [4B-^{3}H]-NADPH was prepared from D-[1-^{3}H(N)] glucose (10–15 Ci/mmol; NEN) by the method of Moran et al. (1984). The resulting radiolabeled coenzyme was purified by HPLC using a Zorbax ODS (Dupont, Boston, MA) column (0.46 × 25 cm) eluted isocratically

with 35% methanol–65% PIC-A reagent (Waters, Millford, MA) at a flow rate of 1 ml/min at ambient temperature. The mass in the eluant was monitored at 280 nm, which allowed both NADPH (standards from Sigma, St. Louis, MO) and NADP to be detected. The time of elution for NADPH was 11.8 min and for NADP was 5.2 min. The peak corresponding to NADPH was collected and the sample lyophilized (using a Hetovac system) to dryness. The specific activity (dpm/μg coenzyme) of the radiolabeled NADPH was determined by HPLC (a standard curve was developed that related increasing amounts of NADPH to increasing change in peak height; detector set at 280 nm) and scintillation counting of the 11.5–12.5 min fraction from the HPLC. In all radiotracer studies the scintillant was Fisher (Pittsburgh, PA) scintiverse BD cocktail. The liquid scintillation counter was a Beckman LS 7000 (Fullerton, CA). The specific activity of the radiolabeled substrates is as follows: [4-^{3}H]-NADPH, 3.2×10^7 dpm/mg; [3-^{3}H]-desmosterol, 8.7×10^7 dpm/mg; [3-^{3}H]-lanosterol, 3.4×10^8 dpm/mg; and [3-^{3}H]-cycloartenol, 1.7×10^8 dpm/mg. [24-^{2}H]-lanosterol was prepared from lanosterol as described (Raab et al., 1968) and purified by HPLC; molecular weight from mass spectroscopy M^+ 427 amu corresponded to the introduction of one deuterium atom in the sterol side chain.

Enzyme Preparation and Assay

Larvae were placed in chilled Ringer's solution, and midguts were removed surgically. Combined midguts from ten larvae were placed in 100 ml of phosphate buffer (pH 7.4) at 4°C and homogenized with an ultra-turrax T-25 tissue homogenizer set on low speed. The homogenate was filtered through cheesecloth and centrifuged at low speed (8,000 rpm) for 15 min. The resulting supernatant was centrifuged using a Beckman Ultra-80 centrifuge at 105,000*g* for 90 min to sediment the microsomes. The resulting pellets (four) were stored in a freezer at –80°C.

Frozen pellets were thawed rapidly by hand and kept in ice before use. To the thawed pellet, dislodged from the centrifuge tube with a glass rod, was added phosphate buffer giving a protein concentration in the suspension of approximately 5.2 mg/pellet. Protein was determined by the modified Lowry method of Schachterle and Pollack (1973) with bovine serum albumin as the standard. We observed that frozen pellets gave about the same activity as fresh pellets, and they could be stored for up to 3 weeks with minimal loss of activity.

A standard microsomal suspension (0.5 ml) from 3-day fifth instar larvae was prepared with 12 μl of [3-^{3}H]-sterol dissolved in phosphate buffer (pH 7.4 and containing 42.5 μM $MgCl_2$ and 27 μM NAD), 12 μl Tween 80 (stock solution, 50 mg/ml ethanol), and 100 μl of NADPH to give a final concentration of sterol of 50 μM and coenzyme of 1 μM and incubated at 37°C for 50 min. The reaction was terminated by the addition of 0.5 ml of 10% methanolic KOH. The resultant neutral lipids, operationally referred to as the nonsaponifiable lipid fraction (NLF), were extracted (3× with 8 ml) with skellysolve F (mixed hexanes from Fisher). The extracts were pooled, the solvent evaporated, and the residue chromatographed by HPLC. In some studies, we added several micrograms of nonradioactive metabolite to the NLF to provide sufficient

mass which could be monitored by UV detection at 205 nm wavelength. The radioactivity eluting from the column corresponding to the sterol substrate and metabolite was measured using the scintillation spectrometer. In HPLC, saturated sterols are difficult to detect (e.g., cycloartanol). Nevertheless, we could predict the correct α_c for cycloartanol and isolate the stanol based on structure–retention time indices we constructed earlier for sterol chromatography in HPLC (Xu et al., 1988; Guo et al., 1995).

RESULTS

Sterol Composition of Larvae, Microsomes, and Diet

The sterol composition of larvae reared on diets supplemented with stigmasterol and sitosterol contained mostly cholesterol (Table 1; Fig. 2). The sterol composition of the diet contained mostly 24-alkyl sterols (phytosterols), whereas the sterol composition of microsomes obtained from 5-day fifth instar larvae (third-day fifth instar larvae contained a similar sterol profile) contained mostly 24-desalkyl sterols (e.g., cholesterol). The NLF from the diet which contained some endogenous sterol was chromatographed by reversed-phase HPLC, and fractions containing sterol were eluted from the column. The HPLC fractions were monitored by GLC and found to contain no additional sterols. A pentacyclic triterpenoid, α-amyrin, was detected in the sterol mixture. This triterpenoid was absorbed along with the phytosterols into the midgut but apparently was neither metabolized nor harmful to growth at the level it occurred in the diet (cf. Svoboda et al., 1995). A novel cholesterol isomer, cholest-7-en-3β-ol (lathosterol), was detected as a minor sterol (Fig. 3, bottom panel) in the microsome-bound sterol mixture, M^+ 384 amu and RRTc on 3% SE-30 packed column of 1.10. The sterols were identified by comparison of their chromatographic properties in GLC and HPLC and mass spectrum with authentic standards. Samples of sitosterol and stigmasterol were recovered from the larvae and examined by ^{1}H-NMR. They were found to be epimerically pure at C-24 (i.e., with an α-oriented ethyl group) (Guo et al., 1995).

24(*R,S*),25-Epiminolanosterol (IL) Effects on Growth and Sterol Metabolism

Insects were reared on increasing concentrations of IL (1–520 ppm) with either sitosterol or stigmasterol as the sterol supplement. As shown in Table 1, IL inhibited maturation and development of larvae (I_{50} of 60 ppm), and inhibited the conversion of phytosterol to cholesterol, much like the physiological and biochemical response of *M. sexta* to 25-azasteroids (Svoboda et al., 1972). Several novel sterols were isolated from the IL-treated insects. Figure 4 shows the total ion current (TIC) chromatogram of the sterol fraction from stigmasterol reared IL-treated larvae. Seven peaks were observed: peak 1, M^+ 386 (cholest-5-enol; cholesterol); peak 2, M^+ 384 (cholesta-5,24-dienol; desmosterol); peak 3, M^+ 382 cholesta-5,22,24-trienol); peak 4, M^+ 400 (24-methyl cholesterol; campesterol); peak 5, M^+ 412 (24-ethyl cholesta-5,22-dienol; stigmasterol); peak 6, M^+ 382 (cholesta-7,22,24-trienol); and peak 7, M^+ 414 (24-ethyl cholesterol; sitosterol). The mass spectrum of each of the seven sterols matched authentic specimens available to us. The reversed-phase HPLC

TABLE 1. Growth Response and Sterol Composition of *Manduca sexta* Reared on Different Sterol Supplements*

Sterol supplement	Growth response	Sterol (mg) insect	Sterol composition (as % total sterol)	Structure[a]
Sitosterol	3.3 cm/4.0 g (fr. wt.) (3-day fifths)	1.26	*Cholesterol(69)*	1C
			Desmosterol(tr.)	1B
			Campesterol(14)	1F
			Sitosterol(17)	1G
Sitosterol	4.5 cm/4.8 g (fr. wt.) (5-day fifths)	1.82	*Cholesterol(66)*	1C
			Desmosterol(8)	1B
			Campesterol(8)	1F
			Sitosterol(18)	1G
Stigmasterol	4.5 cm/4.6 g (fr. wt.) (5-day fifths)	1.80	*Cholesterol(70)*	1C
			Desmosterol(tr)	1B
			Campesterol(8)	1F
			Sitosterol(8)	1G
			Stigmasterol(11)	1H
			Stigmasta-5,22, 24(28)-trienol(tr)	1J
			Unknown(2)	
Sitosterol + 24 (*R,S*), 25-epimino lanosterol	3.5 cm/5.4 g (fr. wt.) (precococious fifth instar)	1.48	Cholesterol(11)	1C
			Desmosterol(58)	1B
			Campesterol(4)	1F
			Sitosterol(26)	1G
			Fucosterol(tr)	1I
			Lanosterol(tr)	4B
Stigmasterol + 24(*R,S*),25-epiminolanosterol	3.6 cm/4.6 g (fr. wt.) (precocious fourth instar)	0.98	Cholesterol(10)	1C
			Desmosterol(38)	1B
			Cholesta-7,22,24-trien-3β-ol(1)	2A
			Cholesta-5,7,22-24-trien-3β-ol(1)	3A
			22-dehydro-desmosterol(14)	1A
			7-dehydro-cholesterol(2)	3C
			Campesterol(10)	1F
			Stigmasterol(11)	1H
			Sitosterol(13)	1G
[24-^{2}H]lanosterol	5.0 cm/5.1 g (fr. wt.) (5-day fifths)	1.50	*Cholesterol(38)*	1C
			Cholest-7-enol(3)	2C
			Campesterol(10)	1F
			[24-^{2}H]-lanosterol isomer(5)	
			[24-^{2}H]lanosterol(21)	4D
			[24-^{2}H]-24,25-dihydrolanosterol(23)	4E

*Test compounds were added to the diet at 240 ppm, except for [24-^{2}H]lanosterol, which was supplied at 130 ppm, and 24(*R,S*),25-epiminolanosterol was added at 45 ppm with stigmasterol and at 15 ppm with sitosterol.

[a]Structures of sterols are given in Fig. 2.

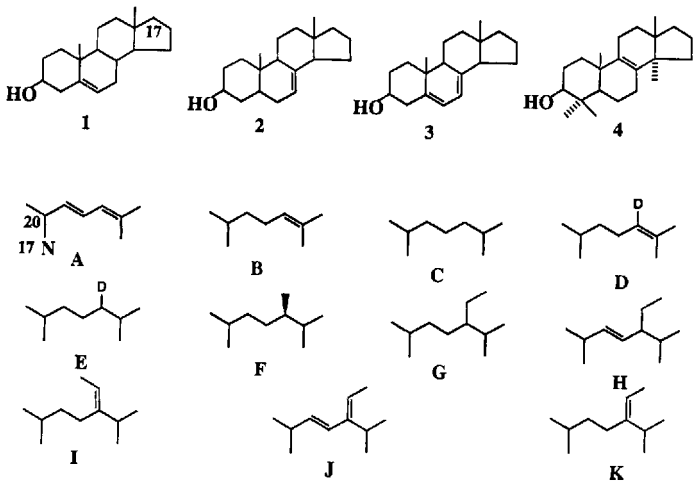

Fig. 2. Structures of sterols identified in this study (nucleus and side chain are given). Systematic nomenclature for the naming of sterols is based on the pairing of a select side chain with the cholestane nucleus (structure 1).

chromatogram of the sterol fraction, eluted from TLC corresponding to 4-desmethyl sterols, showed six peaks (Fig. 4), indicating that one of the seven sterols might be coeluting with another sterol. However, when the sterols were eluted from the HPLC and examined by GC-MS, rather than finding one additional sterol, three additional sterols were detected: cholesta-5,7,22,24-tetraenol (M^+ 380 and other diagnostic ions at 299 (base peak), 273, 269, 207, 147, and 109 amu) which coeluted with cholesta-5,22,24-trienol in HPLC but possessed a slightly longer retention time in GLC, and cholesta-5,7-dienol which eluted as a single component as peak 2 from the HPLC (Fig. 4). Cholesta-5,7-dienol (7-dehydrocholesterol) possessed the same GLC RRTc as desmosterol (see Fig. 4A, C), but it was not evident in the initial monitoring of the GC peaks using the mass detector (see Fig. 4A, C). The apparent failure of the mass detector to uncover the desmosterol isomer was likely because of the isomer's low mass in that GLC peak. The identity of the sterol isomers eluted from the HPLC with molecular weights, M^+ 384, was determined by obtaining a ^{1}H-NMR spectrum on each sample (Fig. 5).

Our ^{1}H-NMR spectral data obtained on the novel cholesta-5,22,24-trienol (Fig. 5) differed slightly from samples synthesized by Hutchins et al. (1969). This was probably due to the improved NMR spectrometers (e.g., having higher field strength) available to us. The identity of fucosterol, in the IL-treated insects fed with sitosterol, was based on its coelution with cholesterol in HPLC (Whatman C_{18} column) and by its characteristic MS fragmentation (M^+ 412 with a base peak of 314 amu). In addition, it coeluted with sitosterol in GLC (3% SE-30) rather than slightly afterwards which is the case for isofucosterol, which is present in the diet (Fig. 6). Fucosterol is thought to be a natural intermediate in the conversion of sitosterol to cholesterol (Svoboda, 1994). The identity of cholesterol, isolated in milligram quantities by HPLC from three separate incubations (sitosterol, stigmasterol, and IL-treated/sito-

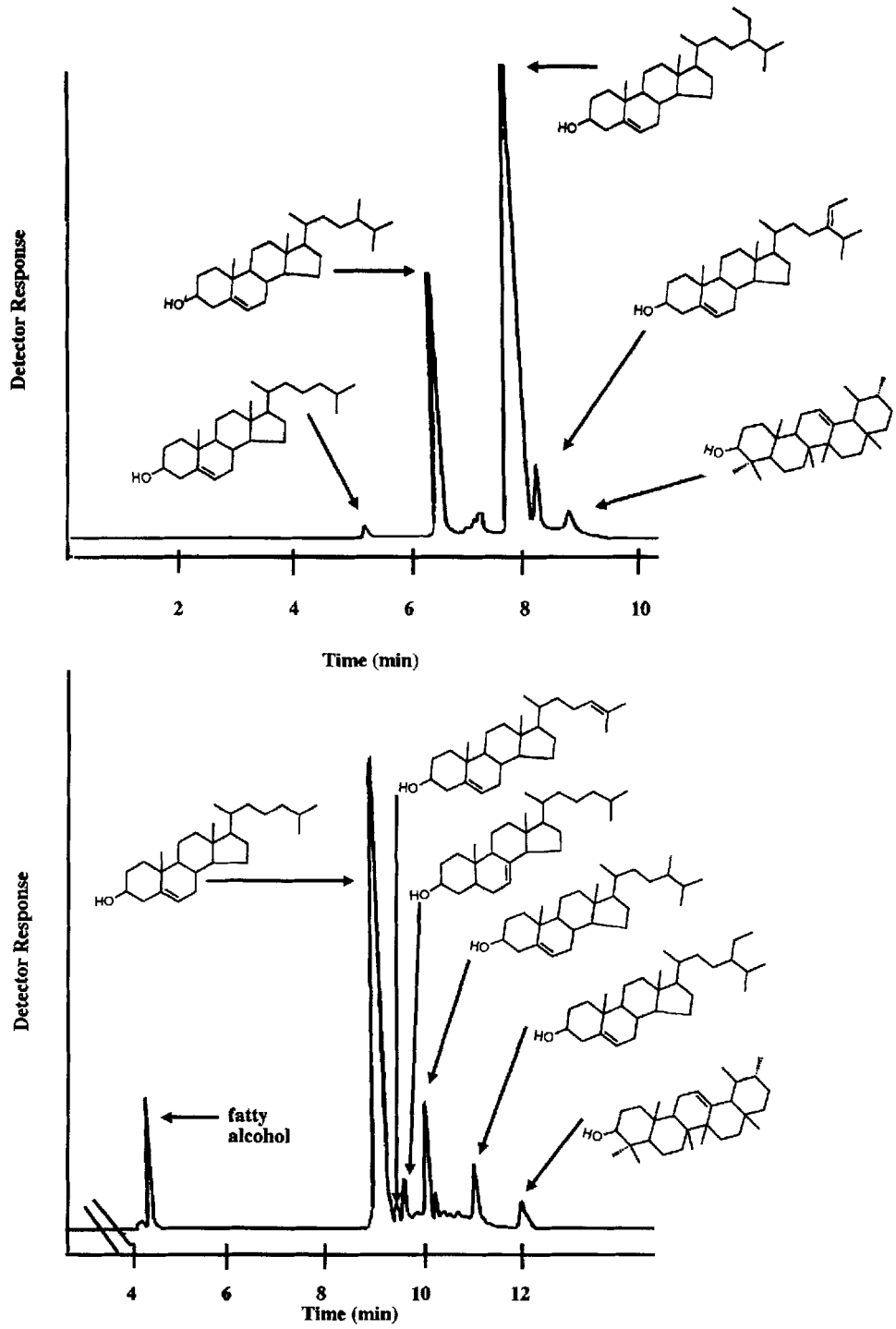

Fig. 3. Gas liquid chromatographic separation of the total sterol fraction of the diet (**top**) and the microsomes from *Manduca sexta* (**bottom**). The structures eluted in the following order: (top) cholesterol(1C), campesterol(1F), sitosterol(1G), isofucosterol(1K), and α-amyrin (pentacycle); (bottom) cholesterol(1C), desmosterol(1B), cholest-7-enol(2C), campesterol(1F), sitosterol(1G), and α-amyrin (pentacycle).

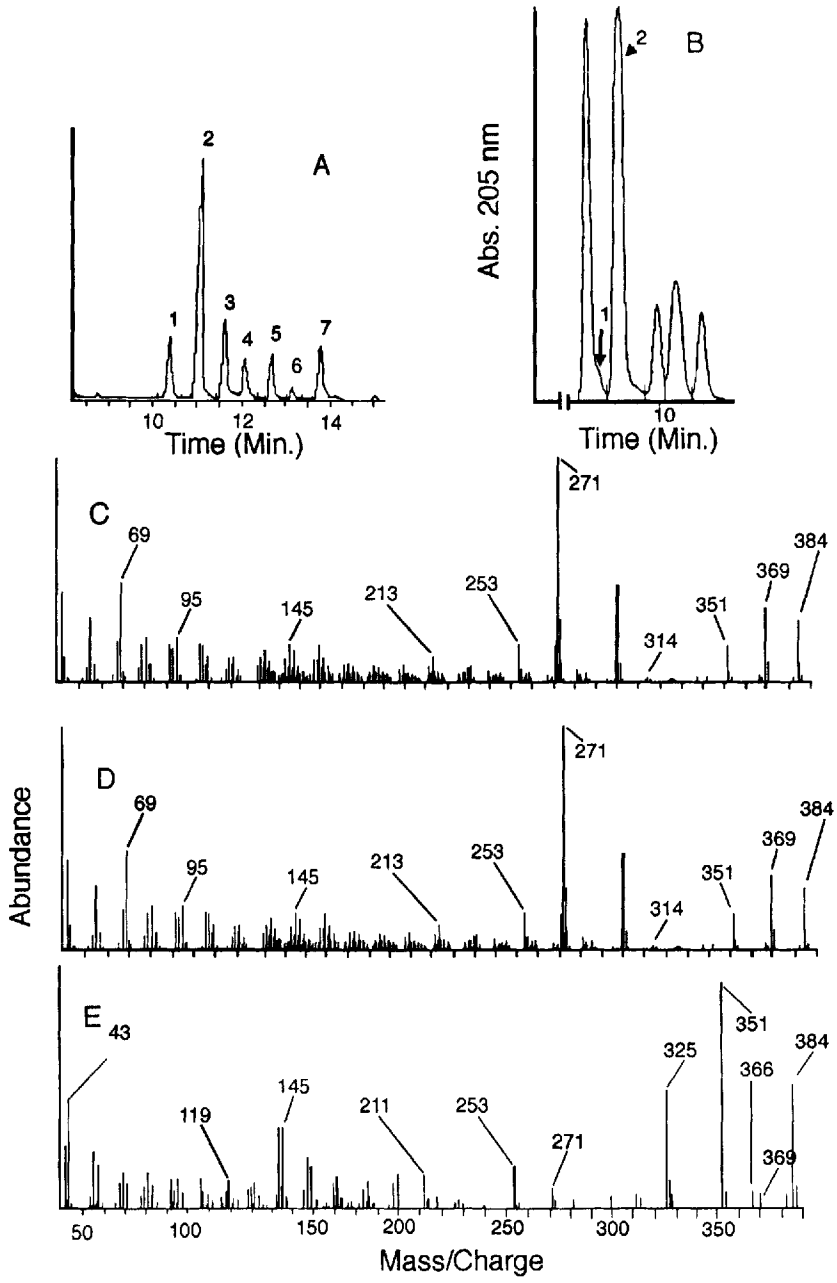

Fig. 4. **A:** Gas liquid chromatographic separation (also referred to as a total ion chromatogram during GC/MS analysis of the 4-desmethyl sterol fraction from *Manduca sexta* reared on stigmasterol and IL. **B:** Reversed-phase HPLC chromatogram (analytical Whatman C_{18} column, eluted at 1.6 ml/min with 4% aq. methanol and operated at 40°C) of the 4,4-desmethyl sterol fraction. **C:** Spectrum derived from the scanning of peak 2 in the top of the peak in A, showing what appears to be a pure sterol. **D,E:** Mass spectra of the sterol samples eluted from the HPLC (B). HPLC peak 1 corresponds to E (7-dehydrocholesterol), and HPLC peak 2 corresponds to D (desmosterol).

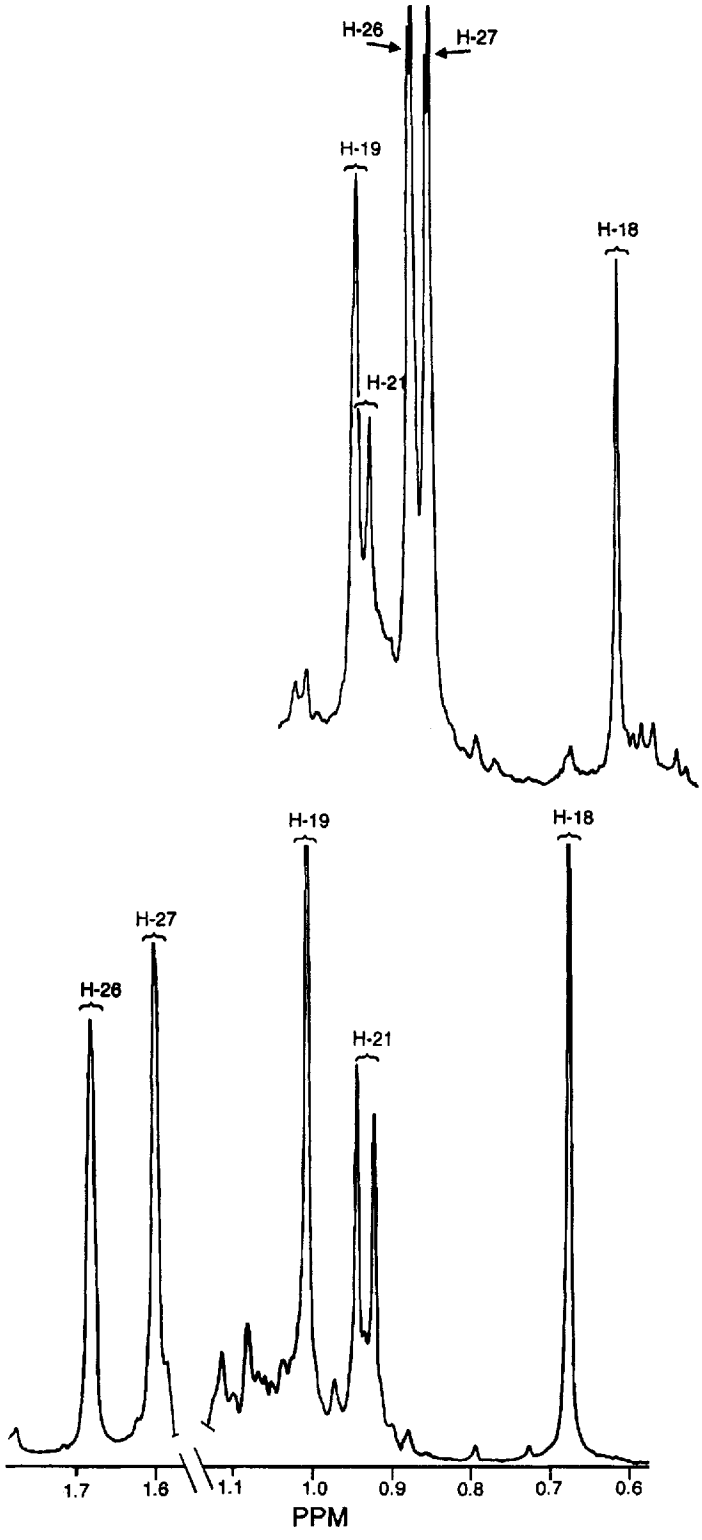

Fig. 5. ^{1}H-NMR spectra of the sterols eluted in the HPLC shown in Fig. 3. The top spectrum corresponds to 7-dehydrocholesterol (H-18 resonates at 0.618 ppm), and the bottom spectrum corresponds to desmosterol (H-18 resonates at 0.679 ppm).

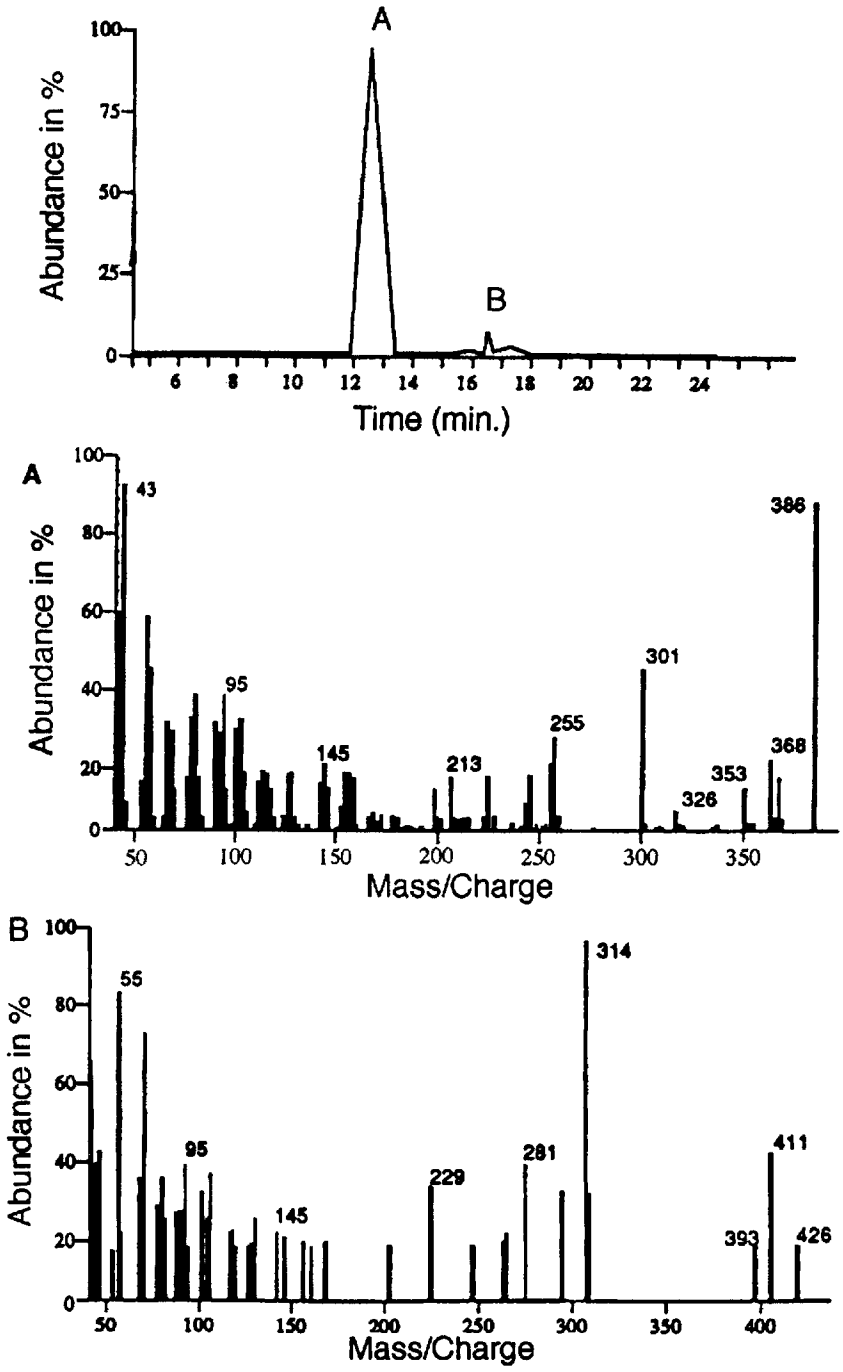

Fig. 6. Total ion current chromatogram (**top**) of the sterol fraction eluted from the HPLC (analytical Whatman C_{18} column) corresponding to a_c of 1.00. **A,B:** Mass spectra for peaks A and B shown in the TLC chromatogram. The sterol fraction was obtained from IL-reared larvae supplemented with sitosterol.

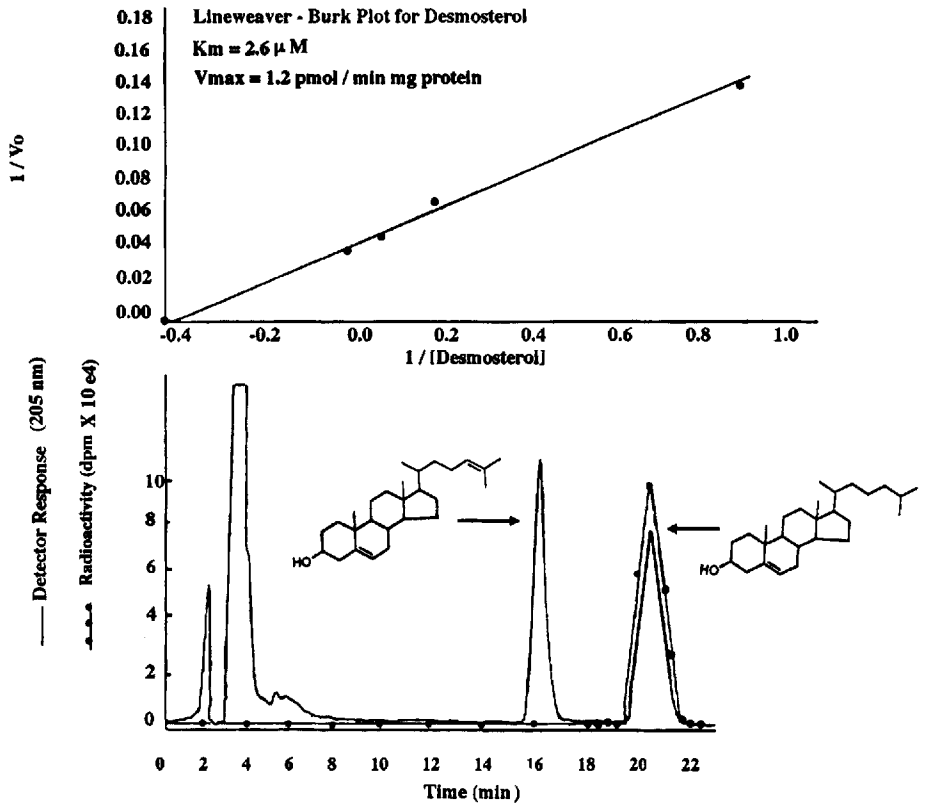

Fig. 7. Lineweaver-Burk plot for desmosterol assayed with the Δ^{24}-sterol reductase (**top**). HPLC chromatogram (**bottom**) and radioactivity profile of the separation of nonradioactive desmosterol from [3-^{3}H] cholesterol (nonradioactive desmosterol from [3-^{3}H] cholesterol (nonradioactive cholesterol was not added to the sample NLF because it was endogenous to the microsomes). The HPLC analysis (TSK gel C_{18} column eluted with methanol/isopropanol 4/1) is the second HPLC separation of desmosterol and cholesterol; the first chromatographic system (Zorbax C_{18} column) was used to isolate the product and to serve as a step in radiochemical purification.

sterol supplemented diets), was confirmed by comparison of each of the sample's MS and ^{1}H-NMR spectra with that of an authentic specimen. Similarly, the identity of the desmosterol which accumulated in the IL-treated insects was confirmed by ^{1}H-NMR. The presence of the Δ^5-bond was easily detected through the chemical shift for the H-6 proton resonating in the olefinic proton region at δ 5.37 ppm.

Studies on Δ^{24}-Sterol Reductase Activity

From the midgut was prepared a microsome-bound enzyme that transformed desmosterol to cholesterol (Fig. 7). The same enzyme preparation transformed lanosterol and cycloartenol to 24,25-dihydrolanosterol (Fig. 8) and cycloartanol, respectively (data not shown). The apparent K_m for the three

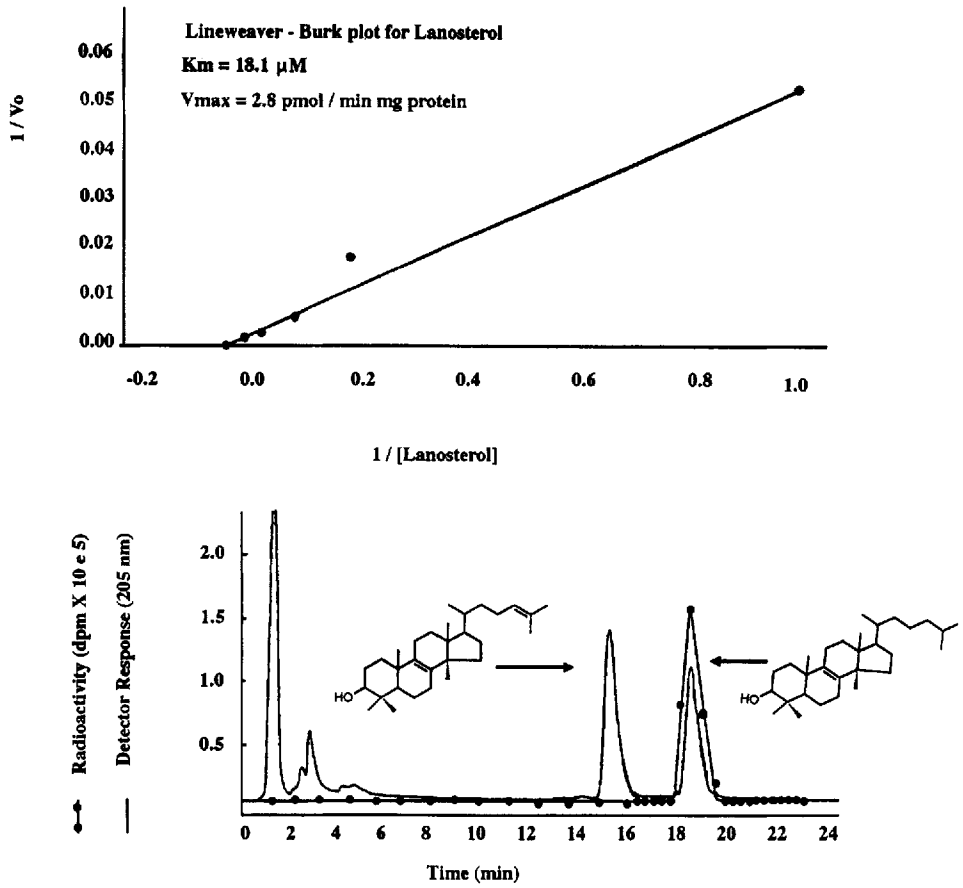

Fig. 8. Lineweaver-Burk plot for lanosterol assayed with the Δ^{24}-sterol reductase (**top**). HPLC chromatogram (**bottom**) and radioactivity profile of the separation of nonradioactive lanosterol from [3-^{3}H]-24,25-dihydrolanosterol (nonradioactive 24,25-dihydrolanosterol was added to the sample NLF). This HPLC analysis (TSK gel C_{18} column eluted with methanol/isopropanol, 4/1) is the second HPLC separation of lanosterol and 24,25-dihydrolanosterol; the first chromatographic system (Zorbax C_{18} column) was used to isolate the product and to serve as a step in radiochemical purification.

substrates was 3 µM (desmosterol), 18 µM (lanosterol), and 33 µM (cycloartenol), whereas the V_{max} values were similar. Clearly, catalytic competence (V_{max}/K_m) was more efficient with desmosterol as substrate for the Δ^{24}-sterol reductase. The radioactive sterol metabolite (from ten pooled assays in the [3-^{3}H]-desmosterol incubation and 20 pooled assays from the [3-^{3}H]-lanosterol and [3-^{3}H]-cycloartenol incubations) was identified by measuring the mass (NLF samples were isotopically diluted with 500 µg of nonradioactive carrier which was thought to be the metabolite) and radioactivity at each chromatographic step that is designed to produce a radiochemically pure sample: first migration on TLC, then, after elution of the appropriate band from TLC, chromatography using two HPLC systems (Zorbax and TSK C_{18} columns). In each experiment the starting activity of the pooled samples was sufficient,

Fig. 9. Mass spectrum (**top**) and ^{1}H-NMR spectrum (**bottom**) of the [24-^{2}H]24,25-dihydrolanosterol sample isolated from *Manduca sexta* larvae reared on [24-^{2}H]lanosterol.

approximately 1 x 10^{5} dpm, for us to adequately measure the specific activity of the radiolabelled metabolite at each chromatographic step of its purification. In none of the experiments was the specific activity of the final product less than 10% of the starting specific activity of the sample applied to the TLC plate.

Studies on the Mechanism of Sterol 24,25-Reduction

M. sexta larvae fed [24-^{2}H]lanosterol were harvested in the fifth instar with no apparent effects on development at the concentration tested. The deuterated labeled substrate and two other deuterated 4,4-dimethyl sterols were isolated from six prepupae (Table 1). We made a careful examination of the 4-monomethyl and 4-desmethyl sterol fractions eluted from the TLC plate by GC-MS, and no deuterated sterols could be detected. Thus, *M. sexta* does not transform lanosterol to cholesterol, as reported earlier using [2-^{3}H]lanosterol fed to *M. sexta* (Svoboda et al., 1995). In the purification of the 4,4-dimethyl sterols, we used an analytical Whatman reversed-phase C_{18} column operated at 40°C with 4% aq. MeOH as the eluant (Xu et al., 1988). Two deuteriated sterols were isolated from the HPLC fraction corresponding to authentic lanosterol. Both deuterated sterols possessed similar molecular weights (M^+ 427 amu) but different retention times in GLC (3% SE-30); one possessed an RRT_c of 1.42, and the other possessed and RRT_c of 1.63 (our starting deuterated compound, [24-^{2}H]lanosterol). The identity of the unknown metabolite is under investigation. From the HPLC fraction corresponding to 24,25-dihydrolanosterol was isolated a single deuterated compound, identified as [24-^{2}H]24,25-dihydrolanosterol by its movement in TLC, GLC (RRT_c of 1.50), its fragmentation in MS (M^+ 429, which corresponds to a one mass unit increase from the molecular weight of 24,25-dihydrolanosterol), and ^{1}H-NMR spectrum (Fig. 9).

Inhibition of reduction of the sterol side chain by IL was demonstrated by the lack of conversion of lanosterol to 24,25-dihydrolanosterol; the I_{50} was 2 μM (data not shown). We chose to use lanosterol rather than desmosterol in this experiment since the substrate and inhibitor would possess the same structural features in the nucleus. We have studied other inhibitors (e.g., 25-azacholesterol) and other sterol substrates (e.g., desmosterol) with similar results. These observations will be communicated elsewhere.

A microsome-bound Δ^{24}-sterol reductase was incubated with freshly prepared 4S-tritium–labelled NADPH and desmosterol. The sterol reaction product was purified from the unlabeled sterol substrate by isotopic dilution of the NLF with 500 μg of cholesterol and then submitting the sample to TLC and HPLC chromatography. As shown in Figure 10, a peak of radioactivity was coincident with the mass of cholesterol in HPLC chromatography, indicating that the tritium was introduced into the sterol side chain to neutralize the carbenium ion introduced at C-25 which was created following proton attack at C-24.

Metabolism of 24(*R,S*),25-Epiminolanosterol (IL)

When IL was fed to the hornworm, lanosterol was detected in the sterol mixture which was recovered from the HPLC fraction corresponding to cholesterol (lanosterol coelutes with cholesterol on the Whatman C_{18} column [Xu et al., 1988]) (cf. Fig. 6). We considered the possibility that lanosterol may have come from laboratory-introduced contamination or from the artificial diet. Therefore, to determine more precisely whether IL was converted to lanosterol by *M. sexta*, we fed larvae radiochemically pure [2-^{3}H]IL. Table 2

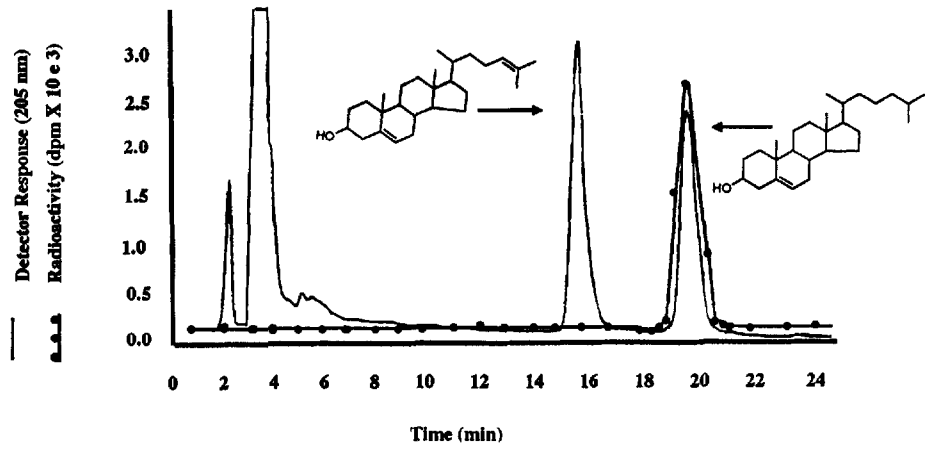

Fig. 10. HPLC chromatogram and radioactivity analysis of sterol fraction from enzyme assay with desmosterol and [4S-^{3}H] NADPH. HPLC column was packed with TSK gel and eluted with methanol/isopropanol, 4/1, at ambient temperature.

shows the sequence of steps involved in the TLC and HPLC purification of the [2-^{3}H]lanosterol. Some loss of radioactivity occurred during the preparative workup of the sample by HPLC (Fig. 11). The radiochemical purity of the tritiated lanosterol sample was demonstrated in the next experiment. Several samples of [2-^{3}H]lanosterol C-3 acetate were recovered from the

TABLE 2. Transformation of [2-^{3}H]24(*R,S*),25-Epiminolanosterol (15 ppm in diet) to Lanosterol by *Manduca sexta*

Material	Level of sterol and radioactivity
Total activity added	1.8×10^8 dpm
Total number of prepupae extracted	5
Total culture volume (diet)	0.4 l
Total mass of insects	26.8 g
Total sterol recovered	9.1 mg
Lanosterol recovered	12 μg
Weight of nonsaponifiable lipid fraction (NLF)	376 mg
Total radioactivity of aqueous layer	1.2×10^6 dpm
Total radioactivity of organic layer (NLF)	2.12×10^6 dpm
Total radioactivity of TLC band corresponding to 4,4-desmethyl sterols	1.1×10^3 dpm[a]
Total radioactivity of TLC band corresponding to 4,4-dimethyl sterols	3.4×10^4 dpm[b]
Total radioactivity at origin	1.10×10^6 dpm[c]
Total radioactivity of HPLC purified lanosterol acetate	5.40×10^3 dpm

[a]When the sterol was eluted from the TLC plate and injected into the HPLC, all the radioactivity eluted in the first 5 min, indicating that the ratioactivity corresponded to radioactive drift on the TLC plate or to autooxidized lanosterol.

[b]HPLC-purified lanosterol was isotopically diluted with 3 mg of nonradioactive lanosterol, and the sample was acetylated at C-3 with acetic anhydride/pyridine.

[c]IL remains at the origin in the system used here to develop the TLC plates (benzene/ether, 85/15,v/v).

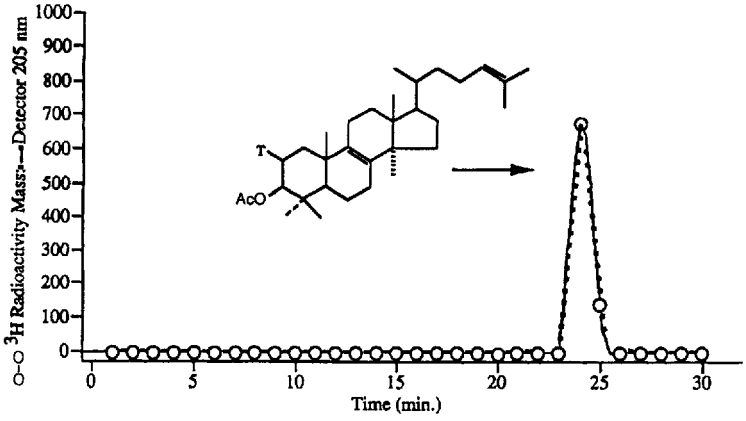

Fig. 11. HPLC chromatogram and radioactivity analysis of 3-acetyl-[2-^{3}H]lanosterol. The sample was isotopically diluted with nonradioactive lanosterol and acetylated at C-3 with acetic anhydride in pyridine. The chromatographic system was a Whatman C_{18} semipreparative reversed-phase column eluted with methanol at 1 mL/min and operated at ambient temperature.

semipreparative reversed-phase HPLC column, pooled, and then isotopically diluted with 50 mg of lanosterol C-3 acetate. The sample was recrystallized several times from hexane and methanol, and no significant loss in the sample's starting specific activity, 101 dpm/mg, was observed. In contrast to our earlier observation that lanosterol was converted to 24,25-dihydrolanosterol (Svoboda et al., 1995), in this study lanosterol was the only labeled neutral sterol recovered from the larvae. The inability to detect 24,25-dihydrolanosterol in the IL-treated larvae is consistent with the expectation that the inhibitor blocks the conversion of lanosterol to 24,25-dihydrolanosterol.

DISCUSSION

The sterol specificity of the Δ^{24}-sterol reductase reported here is to the best of our knowledge the first examination of the K_m and V_{max} of the enzyme from any source. Previous studies of the cell-free Δ^{24}-sterol reductase from mammals (Paik et al., 1984; Gibbons et al., 1971; Kienle et al., 1973; Wilton et al., 1970) and insects (Svoboda et al., 1969; Fujimoto et al., 1985, 1991) were focused on the development of an assay system that could be used to study the mechanism of reduction of the 24,25-bond and the influence of inhibitors on saturation of the double bond. Prestwich et al. (1985) studied the fucosterol epoxide lyase (the enzyme that performs C-24 dealkylation to give the 24,25-bond) from *M. sexta*. Their experimentally determined apparent K_m and V_{max} values for the lyase were several orders of magnitude less than the values we report here for the 24,25-reductase, indicating that the catalytic competence of the lyase was less efficient compared with the 24,25-reductase from the same insect.

Our results with desmosterol, lanosterol, and cycloartenol show that the Δ^{24}-sterol reductase discriminates structural features in the sterol molecule. Of the sterols tested, desmosterol (a sterol which lacks the 4,4-dimethyl group)

was the preferred substrate for catalysis. In an earlier study, feeding [2-^{3}H]lanosterol to *Manduca sexta*, we found the insect acted on one functional site in the molecule (i.e., to saturate the side chain 24,25-bond) (Svoboda et al., 1995). Additionally, cycloartenol was transformed to cycloartanol, but the in vivo transformation of cycloartenol was found to be much less efficient than with lanosterol. The in vivo results agree with the sterol specificity observed by the Δ^{24}-sterol reductase. The results of this study (including the feed with [24-^{2}H]lanosterol where we found no deuterated sterols other than 4,4-dimethyl sterols) and our earlier study feeding [2-^{3}H]lanosterol (Svoboda et al., 1995) indicate that *M. sexta* may accumulate 9β,19-cyclopropyl and Δ^8-sterol intermediates which are found naturally in host plants but will not convert them to Δ^5-sterols, such as cholesterol. Although lanosterol and cycloartenol are inert metabolically, the 24,25-bond was reduced by the insect. These observations contrast with those of Ritter (1986), who suggested lanosterol could be transformed to cholesterol by a related Lepidopteran, *Heliothis zea.*

The ability of *M. sexta* to exhibit sterol specificity and to reduce the 24,25-bond of lanosterol and cycloartenol was of special interest to us. There are several reports inferring lanosterol is flat and cycloartenol is bent conformationally; these conformational differences in the substrate are thought to affect catalytic competence of enzymes that act on the sterol substrate (see reviews by Bloch, 1983; Ourisson, 1994). Thus, as a first approximation, bent sterols produced by plants should not enter the phytosterol transformation pathway of insects—although they may be absorbed as recently demonstrated (Ba et al., 1995; Corio-Costet et al., 1989). Our studies on the conformation of cycloartenol (and related 9β,19-cyclopropyl sterols) in solution and in the solid state (Nes et al., 1988, 1991), the chromatographic and membrane properties (Xu et al., 1988; Nes et al., 1993), and the binding properties of these sterols to plant enzymes (Janssen and Nes, 1992) clearly show that cycloartenol exists in the flat conformation under physiological conditions. Therefore, we expect that Δ^8-sterols and 9β,19-cyclopropyl sterols will be recognized in a similar manner by *M. sexta* larvae. Whereas some difference was apparent in the K_m values for lanosterol and cycloartenol, this difference is likely to be the result of subtle conformational differences that occur in nuclear strain which affects the planar conformation, which affects the tilt of the 3-OH group and the 17(20) bond. Variation of these sterol features is known to affect the development of a productive sterol-protein complex (Janssen and Nes, 1992).

Incubation with [24-^{2}H]lanosterol and [4S-^{3}H]NADPH confirmed that the mechanism of reduction of the sterol 24,25-bond proceeds via the addition of two hydrogens; one of the hydrogen atoms likely comes from the medium (water) and is added to C-24; the other comes from NADPH and is added to C-25. The ability of IL to prevent saturation of the 24,25-bond is consistent with the inhibitor acting as a high energy intermediate anlog of the reaction progress, interfering with the formation of the C-25 carbenium ion generated by the activated complex involving desmosterol, normally. We have studied the effect of IL on reduction of the 24,25-bond in cancerous cultured rat hepatoma cells (Popják et al., 1989). As we found in the insect, IL was found to be a potent inhibitor of sterol 24,25-reduction and growth of cultured cancer cells.

Particularly noteworthy is the observation that when [2-^{3}H]IL was fed to *M. sexta*, a neutral sterol was recovered from the larvae, [2-^{3}H]lanosterol. Presumably, the inhibitor was attacked by an H^+ species analogous to the native substrate, after which it was transformed to 25-amino lanosterol. The latter N-sterol was then deaminated and transformed to lanosterol. We observed a similar transformation of IL by the fungus *Gibberella fujikuroi* (Nes et al., 1989). Additional kinetic studies are warranted to define the enzyme-activated properties of the inhibitor. Our results with IL differ from those of Fujimoto et al. (1991), who demonstrated the inability of the related aziridine-sterol, 24(28)-iminofucosterol, to inhibit 24,25-reduction of the sterol side chain, although it inhibited C-24 dealkylation. It is important to note that IL possessed the lanosterol skeleton and that it was the first rationally designed inhibitor of insect phytosterol metabolism designed so as not to mimic the cholesterol nucleus. Studies are in progress to modify in much greater detail the structure of the inhibitor and to evaluate the modifications in vivo and in vitro.

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